REMARKS

Reconsideration and allowance are respectfully requested.

Claims 6, 13, 16-18, 25, 29-30 and 34-38 are pending.

Statement of the Substance of the Interview

The undersigned acknowledges the courtesy extended by the Examiner during the interview on January 27, 2011. Proper construction of the independent claims was discussed, along with the different scope of protection provided by claims 6, 25 and 30. Differences between "neuroprotection" required by Applicants' claim 6 and Bertilsson's neurogenesis were also discussed. Most of the interview involved the inventors' detailed reasons why Gas6 and protein S would not have been considered interchangeable as functional equivalents in view of the prior art. Although no agreement was reached on allowance of the present claims, Applicants promised to provide the latter arguments and supporting evidence in their next response (see below). The foregoing is Applicants' summary of the interview. If anything else is required to complete the record, do not hesitate to contact the undersigned.

35 U.S.C. 103 – Nonobviousness

A claimed invention is unpatentable if the differences between it and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art. *In re Kahn*, 78 USPQ2d 1329, 1334 (Fed. Cir. 2006) citing *Graham v. John Deere*, 148 USPQ 459 (1966). The *Graham* analysis needs to be made explicitly. *KSR Int'l v. Teleflex*, 82 USPQ2d 1385, 1396 (2007). It requires findings of fact and a rational basis for combining the prior art disclosures to produce the claimed invention. See id. ("Often, it will be necessary for a court to look to interrelated teachings of multiple patents . . . and the background knowledge possessed by a person having ordinary skill in the art, all in order to determine whether there was an apparent reason to combine the known elements in the fashion claimed by the patent at issue").Thus, a prima facie case of obviousness requires "some rationale, articulation, or reasoned basis to explain why the conclusion of obviousness is

correct." *Kahn* at 1335; see *KSR* at 1396. A determination of prima facie obviousness also requires a reasonable expectation of success. See *In re Rinehart*, 189 USPQ 143, 148 (C.C.P.A. 1976).

Claims 6, 13, 16-18, 25, 29-30 and 34-38 were rejected as allegedly obvious over Bertilsson et al. (US 2003/0165485) in view of Hung (US 2003/0060415). Applicants traverse for the reasons of record.

Bertilsson discloses the "potential therapeutic use" of protein S signaling in a method of influencing adult neural stem cells and progenitor cells to produce progeny. Thus, the document specifically teaches that the desired result is producing progeny cells that will replace or supplement the cells lost to disease. See, for example, paragraphs [0018] to [0021]. The document does not contain any explicit or implicit teaching that administration of protein S to a subject would provide neuroprotection as required by Applicants' claim 6 because the present invention relies on the effect of protein S on preventing cell death or apoptosis.

The '485 application does not make obvious the present claims. Specifically Bertilsson's examples contain data showing expression of Reelin and its receptors, Gas6 and its receptors, in vitro experiments to define the therapeutic potential of Gas6 and its receptors, the production and purification of human Gas6, and in vivo analysis of Gas6's effect on neural proliferation. But the '485 application is silent on neuroprotective activity of protein S. One of ordinary skill in the art would not readily accept Bertilsson's argument at paragraph [0061] that Gas6 and protein S could serve as functional equivalents:

> Interestingly, Gas6 displays significant amino acid sequence identity to Protein S, an additional member of the vitamin K-dependent family and an important co-factor in the inhibition of the blood coagulation cascade, suggesting possible functional similarities between these two proteins (Manfioletti et al., 1993). In support of this model, Protein S has been proposed to serve as an additional ligand for Tyro3 (Stitt et al., 1995).

The skilled artisan would have been aware of the art-known rejection of this concept that protein S would have the same biological activities as Gas6. For example, Nagata et al. (J. Biol. Chem. 271:30022-30027, 1996) reported:

> Although Stitt et al. reported that human or bovine protein S bound to murine Sky (Tyro3), intraspecies (human-human) ligand-receptor interactions of protein S and Sky could not be detected (Ohashi et al., 1995; Godowski et al., 1995).

> Hence, it is probable that Gas6, but not protein S, is a ligand for Sky, and Gas6 may also be a common ligand for Axl and Sky, two related receptors.

Nagata et al. (1996) also concluded:

> The inability of intraspecies (human-human) ligand-receptor interactions between protein S and Sky suggested that protein S could not function as a natural ligand for Sky. Thus, Gas6, but not protein S, seems to be the ligand for the two related receptors, Axl and Sky.

Thus, the skilled artisan would have known of the contradictory scientific literature and their teachings that it would not have been obvious in the art to use protein S and Gas6 as functional equivalents for their biological activities as alleged by Bertilsson.

The Examiner acknowledged that "there had been some controversy in the mid-1990s as to whether or not protein S activates Sky" (page 4 of the Office Action mailed December 30, 2009). He cited Lan et al. (Blood 95:633-638, 2000) for showing Tyro3/Sky is activated by protein S. Although no direct interaction was shown between protein S and Tyro3, Lan concluded, "Our current study also confirmed that interspecies ligand-receptor interaction occurs between protein S and Tyro3" (emphasis added). Even the authors' optimistic view of their results failed to teach or suggest that human protein S would act as a ligand for human Tyro3. Therefore, Lan fails to support what was being alleged in the Office Action: there is no evidence that human protein S and human Sky interact as ligand and receptor. Moreover, the complicated culture system used in Lan would not permit one of ordinary skill in the art to make any conclusion about direct ligand-receptor interactions.

During the interview on January 27, 2011, Applicants cited evidence supporting the finding that Gas6 and protein S are not interchangeable. Attached for the Examiner's consideration are Godowski et al. (Cell 82:355-358, 1995) and Angelillo-Scherrer et al. (Nature Med. 7:215-221, 2001).

Godowski discusses Regeneron's proposal that protein S is an additional ligand for Tyro3 (see Stitt et al., 1995). They disagree with Stitt's earlier conclusion because human Gas6, but not human protein S, is a ligand for human Tyro3. This contradiction is also found by Ohashi et al. (J. Biol. Chem. 270:22681-22684, 1995), who conclude that human protein S is not a ligand for human Sky too.

Angelillo-Scherrer, published almost contemporaneously with Applicant's priority filing, show that a knock-out mouse without functional Gas6 has a different phenotype as compared to a knock-out mouse without functional protein S. Deletion of the protein S gene, but not the Gas6 gene, is embryonically lethal. Moreover, other studies find that protein S is antithrombotic in mice whereas Gas6, by binding platelet receptors, is prothrombotic. Thus, these studies fail to support interchangeability between the Gas6 and protein S, or that the two proteins share similar biologic activity for neuroprotection.

Therefore, one of ordinary skill in the art would not found obvious the neuroprotective activity of protein S as discovered and claimed by Applicants because protein S and Gas6 are not functionally equivalent for the biological activity of activating Sky. The evidence taken as a whole does not support Bertilsson's contention that protein S would have all of Gas6's biological activities (including Sky/Tyro3 activation).

One of ordinary skill in the art would also not have had a reasonable expectation from the prior art that Applicants' claimed neuroprotection of cells or effective treatment after neurotrauma and stroke would be achieved in a subject without also administering protein C or activated protein C (APC).

Further, it was known in the art that agents promoting neurogenesis (e.g., stroke-induced neurogenesis as discussed in the '485 application) would not necessarily be neuroprotective. For example, Zhang et al. (Stroke 33:2675-2680, 2002) showed that sildenafil will robustly affect post-stroke neurogenesis and promotes functional recovery related to neurogenesis, but sildenafil is not neuroprotective and does not reduce brain infarction after stroke. Similarly, statin (Chen et al., Ann. Neurol. 53:743-751, 2003) and erythropotein (Wang et al., Stroke 35: 1732-1737, 2004) enhanced post-ischemic angiogenesis and neurogenesis, but they are not neuroprotective and do not reduce brain infraction after stroke. Therefore, from Bertilsson's disclosure, one of ordinary skill in the art would not conclude that it would have been obvious with a reasonable expectation of success for agents that promote neurogenesis to be neuroprotective agents too. Neurogenesis and neuroprotection are two distinct processes as acknowledged in the art.

Hung discloses the treatment of coronary conditions and cardiovascular indications, which were defined as referring to a diagnosis or presumptive diagnosis of cardio-

vascular disease and to a condition that affects the heart (paragraph [0070]). The document refers to "treatment" as the treatment of cardiovascular disease (see paragraph [0071]). The '415 application does not refer to treatment of the brain or prevention of neural cell death. A therapeutic agent (e.g., protein S) is specifically taught to be directly delivered into the pericardial space. But the '415 application does not contain any explicit or implicit teaching that administration of human protein S to a human subject would provide neuroprotection as required by Applicants' claim 6 because the present invention relies on the effect of protein S in the brain. As regards Applicants' claims 25 and 30, there was no disclosure in the '415 application that treatment of neurotrauma or stroke would be achieved without also administering protein C or APC. Nowhere in the '415 application was it disclosed or made obvious that no protein C or APC is administered, there is no deficiency of protein S activity in the subject, or any of the recited results of treatment could be achieved with a reasonable expectation of success.

Finally, the present claims are not obvious over Hung because it teaches away from Applicants' claimed invention by limiting the invention disclosed in the '415 application to delivery of the therapeutic agent to the pericardial space. Applicants' claims do not require delivery of human protein S to the patient's pericardial space.

These deficiencies of the Bertilsson and Hung applications are not remedied by combining them. Alone or in combination, the cited applications fail to teach or suggest any beneficial effect of administering protein S on neuroprotection when no protein C or APC is administered to a subject. Bertilsson relates to the beneficial effect of protein S on neural stem cells and neural progenitor cells, not differentiated neural cells. Hung is concerned with the treatment of coronary and cardiovascular conditions, instead of the cell death or apoptosis in the nervous system that is prevented by using Applicants' invention. Specifically, the cited applications contain no disclosure of using protein S to provide neuroprotection (claim 6), to treat neurotrauma (claim 25), or to treat stroke (claim 30) as required by Applicants' invention. There was also no reasonable expectation that administration to a human subject of human protein S would provide "neuroprotection after brain injury caused by at least cerebral ischemia, hypoxia, re-oxygenation, or a combination thereof" (claim 6), an effective treatment after neurotrauma (claim 25),

or an effective treatment after stroke (claim 30). Further, the cited applications contain no reasonable expectation that those beneficial results would be successfully achieved without administering protein C or APC.

Bertilsson and Hung fail to disclose or make obvious a method of treatment using human protein S that does not also administer protein C or APC. It also would not have been obvious to treat human subjects in accordance with Applicants claimed invention when such patients had no deficiency of protein S activity (dependent claims 13, 29 and 34). Moreover, none of the results of the claimed method of treatment recited in dependent claims 17-18 and 35-38 would have been obvious to one of ordinary skill in the art when the claimed invention was made. Therefore, the present claims are not obvious over Bertilsson and Hung.

Withdrawal of the Section 103 rejection is requested because the claims would not have been obvious to one of ordinarily skill in the art when this invention was made.

Conclusion

Having fully responded to the pending Office Action, Applicants submit that the claims are in condition for allowance and earnestly solicit an early Notice to that effect. The Examiner is invited to contact the undersigned if additional information is required.

Respectfully submitted,

NIXON & VANDERHYE P.C.

By: /Gary R. Tanigawa/
Gary R. Tanigawa
Reg. No. 43,180

901 North Glebe Road, 11th Floor
Arlington, VA 22203-1808
Telephone: (703) 816-4000
Facsimile: (703) 816-4100

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Matters Arising

Reevaluation of the Roles of Protein S and Gas6 as Ligands for the Receptor Tyrosine Kinase Rse/Tyro 3

Recently, Stitt et al. (1995) reported that protein S (PS), but not Gas6, is a potent ligand for the receptor tyrosine kinase known as Rse, Tyro 3, Brt, Sky, and Tif (hereafter referred to as Rse/Tyro 3). PS is an abundant serum protein previously characterized as an essential anticoagulant. Gas6, which was identified as a gene whose expression is increased by growth arrest, shares 43% amino acid identity and overall domain organization with PS (Manfioletti et al., 1993). Stitt et al. (1995) based their conclusions on experiments describing interspecies interactions of bovine Gas6 with murine Rse/Tyro 3 (mRse/Tyro 3) and on interactions of bovine and human PS (hPS) with mRse/Tyro 3. Consistent with the results of Stitt et al. (1995), we identified bovine PS as a ligand for human Rse/Tyro 3 (hRse/Tyro 3), and we also found that hPS can act as a ligand for mRse/Tyro 3. However, when we analyzed the more relevant intraspecies interactions, we obtained different results. We found that human Gas6 (hGas6), but not hPS, acted as a potent ligand for hRse/Tyro 3 (Figure 1). The hRse/Tyro 3 we have studied is very likely the true homolog of mRse/Tyro 3 since they share 90% amino acid identity and a similar expression pattern (Mark et al., 1994; Lai et al., 1994).

To characterize the Rse/Tyro 3 ligand, we constructed soluble receptor proteins containing the extracellular domain of either hRse/Tyro 3 or mRse/Tyro 3 fused to the Fc portion of human immunoglobulin G1 (hRse–IgG and mRse–IgG). A similar fusion protein (termed Tyro 3–Fc) was utilized by Stitt et al. (1995) to characterize the binding of bovine Gas6 and hPS to mRse/Tyro 3. We first determined whether hRse–IgG or mRse–IgG differed in its ability to bind to hPS or hGas6 containing an epitope tag that allows for side-by-side comparison of the binding properties of the two proteins. Either hRse–IgG or mRse–IgG was incubated with conditioned medium containing putative ligands. Complexes were captured with protein A (specific for the IgG fusion protein) and visualized with an antibody specific for the epitope-tagged putative ligand. While hGas6 was bound by hRse–IgG, it was not efficiently bound by mRse–IgG (Figure 2). The reciprocal result was obtained in analysis of binding of hPS to hRse–IgG and mRse–IgG; hPS was bound by mRse–IgG but not by hRse–IgG. These results are consistent with an apparent difference in affinity of hPS for human as opposed to mRse/Tyro 3 (hPS prefers mRse/Tyro 3), as well as an inverse difference for hGas6 (hGas6 prefers hRse/Tyro 3).

A second method was used to analyze quantitatively the interactions of hRse/Tyro 3 and mRse/Tyro 3 to hGas6 and hPS. We measured the binding of ^{125}I-hRse–IgG or ^{125}I-mRse–IgG to purified hGas6 or hPS using an assay in which complexes are captured with a method specific for the Gla domain present in hGas6 and hPS (barium coprecipitation) and in which these complexes are quantitated by virtue of the radioactivity in Rse–IgG. While hGas6 bound ^{125}I-hRse–IgG efficiently, giving a half-maximal binding at 8 nM hGas6, binding to ^{125}I-mRse–IgG over the same concentration range was minimal (Figure 3A). In contrast, hPS was capable of binding ^{125}I-mRse–IgG, although at considerably higher concentrations (half-maximal concentration of 90 nM), but bound very little ^{125}I-hRse–IgG (Figure 3B). We then compared the ability of increasing unlabeled hRse–IgG or mRse–IgG to compete for binding with fixed levels of ^{125}I-hRse–IgG to hGas6 (Figure 3C). The binding of ^{125}I-hRse–IgG to hGas6 was competed by low concentrations of unlabeled hRse–IgG (IC_{50} of 0.3 nM), but only by high concentrations of unlabeled mRse–IgG (IC_{50} of 37 nM). In contrast, the binding of ^{125}I-mRse–IgG to hPS was competed by mRse–IgG (IC_{50} of 18 nM) while hRse–IgG did not appear to compete at all (Figure 3D). These studies are entirely consistent with those shown in Figure 2. There was a profound difference in the affinities of hGas6 and hPS for the extracellular domains of mRse/Tyro 3 and hRse/Tyro 3, with hGas6 showing a preference for hRse/Tyro 3 and hPS exhibiting a preference for mRse/Tyro 3. Similar results were obtained when the binding of hPS or hGas6 to receptor fusion proteins was analyzed by molecular interaction analysis using a stirred-cell optical sensor system (data not shown).

hPS was reported to induce Rse/Tyro 3–mediated responses in NIH 3T3 cells. We directly compared the ability of hPS or hGas6 to induce phosphorylation of hRse/Tyro 3 or mRse/Tyro 3 expressed in NIH 3T3 cells. As noted

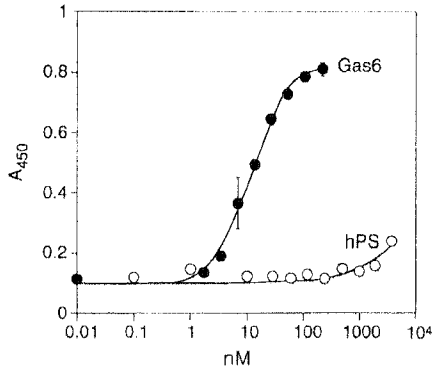

Figure 1. Comparison of Purified hGas6 and Purified Plasma-Derived hPS as Ligands for hRse/Tyro 3

The indicated concentrations of purified recombinant hGas6 (closed circles) or plasma-derived hPS (open circles) (Enzyme Research Laboratories) were tested for ability to induce phosphorylation of hRse/Tyro 3 expressed in CHO cells using an ELISA-based KIRA assay (see Experimental Procedures). Identical results were observed when hRse/Tyro 3 phosphorylation was analyzed by immunoprecipitation using anti-Rse/Tyro 3 antibodies and Western blotting with anti-phosphotyrosine antibodies.

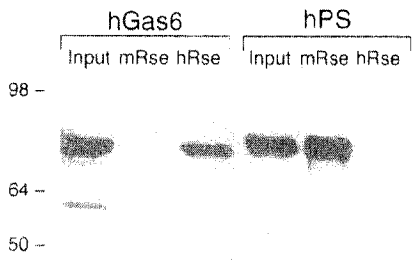

Figure 2. Species-Specific Binding of Epitope-Tagged hGas6 and hPS by hRse–IgG and mRse–IgG

Either hRse–IgG or mRse–IgG was incubated with unfractionated conditioned medium containing epitope-tagged hGas6 or hPS. Complexes were captured with protein A (specific for the IgG fusion protein) and fractionated by SDS–PAGE followed by Western blotting. Bound proteins were detected using the anti-gD antibody specific for the epitope-tagged putative ligand to compare directly the input and bound amounts of recombinant hPS and hGas6. The unfractionated (input) lanes represent 20% of the material used for the precipitation assay. Molecular sizes are indicated on the left (in kilodaltons).

previously (Stitt et al., 1995; our unpublished data), phosphorylation of hRse/Tyro 3 and mRse/Tyro 3 is observed upon treatment of cells with fetal bovine serum (FBS), indicating that both hRse/Tyro 3 and mRse/Tyro 3 expressed in NIH 3T3 cells are functional (Figure 4). Phosphorylation of hRse/Tyro 3 was not observed in response to high levels of either purified hPS or conditioned medium containing recombinant hPS, but was activated by conditioned medium containing hGas6. In contrast, mRse/Tyro 3 was activated by purified hPS and by conditioned medium containing recombinant hPS, but not by medium containing hGas6. The level of hPS required to activate mRse/Tyro 3 in our assays was significantly higher than that reported by Stitt et al. (1995) to activate mRse/Tyro 3 expressed in Rat2 cells. We note that in the studies reported here and by Stitt et al., the levels of recombinant hPS were estimated by comparison to purified hPS on Western blots. The concentration of hPS required to activate mRse/Tyro 3 in the phosphorylation assay reported here is consistent

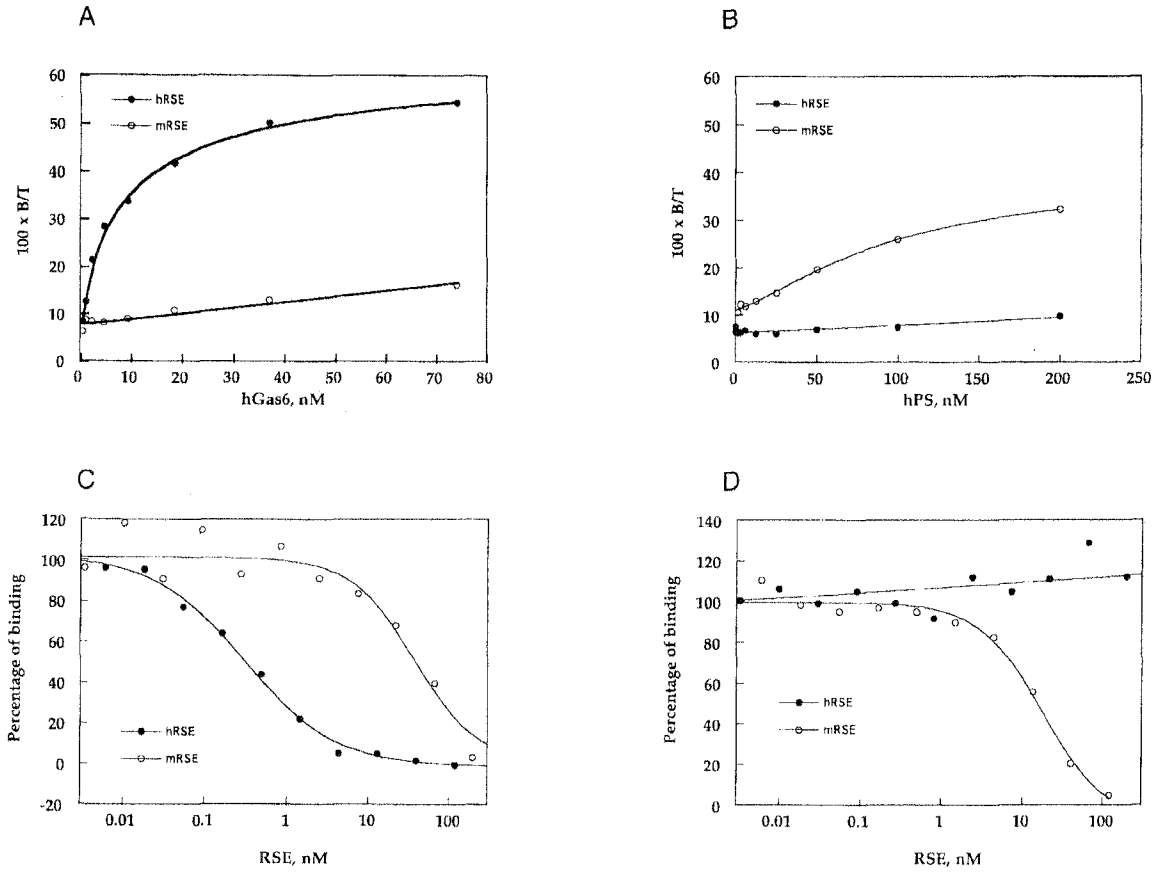

Figure 3. Solution Phase Binding of Recombinant hGas6 and Plasma-Derived hPS to hRse–IgG and mRse–IgG

To measure saturation with ligand, increasing concentrations of hGas6 (A) or hPS (B) were mixed with a fixed amount (50,000 cpm) of ^{125}I-hRse/Tyro 3 or ^{125}I-mRse/Tyro 3, and precipitable radioactivity was measured as described in Experimental Procedures. Binding in (A) and (B) is expressed as 100 × B/T, where T is total radioactivity added and B is radioactivity recovered in the barium pellet. To measure inhibition of binding, concentrations of hGas6 and hPS were chosen to give 100 × B/T of 8–12. A constant amount of hGas6 and ^{125}I-hRse/Tyro 3 (C) or a constant amount of hPS and ^{125}I-hRse/Tyro 3 (D) was mixed with the indicated concentrations of hRse–IgG or mRse–IgG, barium precipitable radioactivity was measured as in Experimental Procedures, and binding was normalized to the percent of that obtained in the absence of added inhibitor with appropriate background subtraction (background of 3%–5% of total counts per minute).

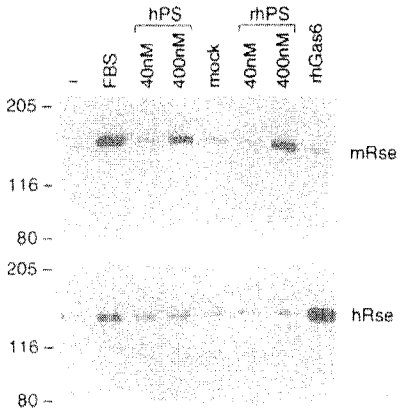

Figure 4. Conditioned Medium from Cells Expressing hGas6 Induces Phosphorylation of hRse/Tyro 3

Phosphorylation of mRse/Tyro 3 is induced by purified hPS or by recombinant hPS (rhPS). NIH 3T3 cells expressing either mRse/Tyro 3 or hRse/Tyro 3 were serum starved and then left untreated (minus) or treated with 20% FBS or the indicated concentrations of hPS purified from human serum or treated with conditioned medium from 293 cells either mock transfected (M) or expressing recombinant hPS or recombinant hGas6 (rhGas6). The amounts of PS and Gas6 in the conditioned medium were estimated by Western blotting and by comparison to purified material of known concentration.

with the relatively modest affinity of hPS for mRse/Tyro 3 that we observe (see Figure 3).

Rse/Tyro 3, along with the related receptors Axl/Ufo and c-Mer, comprises a family of receptor tyrosine kinases whose extracellular domains are reminiscent of neural cell adhesion molecules. Our data demonstrate that hGas6 is a bona fide ligand for hRse/Tyro 3. Interestingly, hGas6 has also been shown to activate human Axl/Ufo (Varnum et al., 1995; our unpublished data), and bovine Gas6 binds to the extracellular domain of murine Axl/Ufo (Stitt et al., 1995). Thus, Gas6 appears to activate multiple receptor signaling pathways.

We also demonstrate a pattern of reciprocal preferences between hRse/Tyro 3 and mRse/Tyro 3 and hPS and hGas6; hRse/Tyro 3 prefers hGas6 over hPS while mRse/Tyro 3 prefers hPS over hGas6. One explanation for the inability of bovine Gas6 to bind to mRse/Tyro 3 (Stitt et al., 1995) is that this interaction may be governed by species differences in receptor–ligand recognition. While our data that hPS binds to and activates mRse/Tyro 3 are in agreement with that of Stitt et al. (1995), the inability of hPS to serve as a ligand for hRse/Tyro 3 brings into question the conclusion that PS is a potent ligand for Rse/Tyro 3. Our results do not rule out the possibility that hPS might activate hRse/Tyro 3 under certain circumstances. For example, modified forms of either the ligand or the receptor might behave differently than the versions we have tested. However, we have tested several sources of hPS (plasma-derived hPS from Enzyme Research Laboratories, Calbiochem, and Celsus Laboratories, as well as recombinant hPS expressed in human 293 and CHO cells) and do not detect significant activation of hRse/Tyro 3. Given its homology to Gas6, a potent ligand for Rse/Tyro 3, and the related receptor Axl (Varnum et al., 1995), PS could yet prove to have a role as a physiologically relevant ligand for a member of the Rse/Axl family.

Paul J. Godowski,* Melanie R. Mark,* Jian Chen,* Michael D. Sadick,† Helga Raab,‡ and R. Glenn Hammonds‡
*Department of Cell Genetics
†Department of Bioanalytical Technology
‡Department of Protein Chemistry
Genentech
South San Francisco, California 94080

Experimental Procedures

Construction and Expression of Recombinant Proteins

hRse–IgG and mRse–IgG were constructed by fusing the sequence encoding amino acids 1–428 of hRse/Tyro 3 or amino acids 1–418 of mRse/Tyro 3 (Mark et al., 1994) to amino acids 216–443 of human IgGγ1. Epitope-tagged gD.hPS or gD.Gas6 were constructed by linking the coding sequences for the gD signal sequence and epitope tag (Mark et al., 1994) by polymerase chain reaction to coding sequences immediately before the first amino acid of mature hGas6 or hPS. Recombinant proteins were expressed in stable 293 cells grown in serum-free medium. For purification, conditioned medium containing receptor–IgG proteins was mixed 1:1 with Pierce ImmunoPure gentle Ag/Ab binding buffer and passed through a 1 ml HiTrap protein A–Sepharose column. Bound proteins were eluted with Pierce ImmunoPure eluting buffer and desalted on a Pharmacia PD10 column into phosphate-buffered saline (PBS). Protein concentration was determined by an anti-Fc enzyme-linked immunosorbent assay (ELISA).

Binding Assays

Immunoprecipitation with IgG fusion proteins was performed by mixing 1 μg of purified mRse–IgG or hRse–IgG with conditioned medium containing 150 ng of hGas6.gD or hPS.gD and 10 μl of protein A–Sepharose CL4B (Pharmacia) in a final volume of 300 μl. Binding was performed at 4°C on a shaker for 12 hr. Complexes were collected by centrifugation and washed three times with 0.5 ml of PBS with 0.1% NP-40. Proteins were separated by reducing SDS–polyacrylamide gel electrophoresis (SDS–PAGE) on Novex 4%–12% minigels, transferred to nitrocellulose, and blotted with monoclonal anti-gD antibody as described previously (Mark et al., 1994).

Iodination of Rse–IgG was performed using Iodobead (Pierce) oxidation of ^{125}I-NaI; specific activity was 250–500 Ci/mmol. Binding studies were performed by incubating samples containing hGas6 or hPS in 25 mM HEPES (pH 7.2), 0.1% BSA, and 0.05% Tween 20 with 50,000 cpm of ^{125}I-mRse–IgG or ^{125}I-hRse–IgG and cold competitor (if any) in a total volume of 100–120 μl. After 45 min at room temperature, 1 ml of a freshly prepared ice-cold suspension of $BaCl_2$ (10 mM) in PBS was added and precipitable radioactivity collected by centrifugation for 4 min at 14,000 × g.

Phosphorylation Assays

CHO cell lines expressing a Rse.gD (which contains a C-terminal epitope tag recognized by antibody 5B6) were established and screened by Western blotting as described previously (Mark et al., 1994). An ELISA-based kinase receptor activation (KIRA) assay (Sadick et al., submitted) was established to measure quantitatively the ligand-induced phosphorylation of Rse/Tyro 3. CHO-Rse.gD cells were treated with potential ligand sources for 15 min, lysed with detergent, and transferred to surfaces coated with antibody 5B6. The degree of phosphorylation was quantitated using a biotinylated anti-phosphotyrosine antibody (4G10; UBI), streptavidin conjugated to horseradish peroxidase (Zymed Laboratories), and a colorimetric peroxidase assay (two-component substrate kit; Kirkegard & Perry). Purified native hPS was obtained from Enzyme Research Laboratories. NIH 3T3 cells expressing hRse/Tyro 3 or mRse/Tyro 3 were established by infection of NIH 3T3 cells with a recombinant ecotropic retrovirus LXSN.hRse or LXSN.mRse encoding hRse/Tyro 3 or mRse/Tyro 3, respectively. Retroviral vectors were used to produce populations consisting of hun-

dreds of thousands of stable clones to avoid the possibility that an individually selected clone might exhibit an aberrant phenotype. Retroviral stocks (titer of ~1 × 10^6 cfu/ml) were prepared, and 1 ml was used to infect 1 × 10^6 NIH 3T3 cells, which were selected as described previously (Pear et al., 1993). NIH 3T3.hRse or NIH 3T3.mRse cells were washed once in PBS and then cultured in high glucose DMEM without serum for 3 hr prior to treatment. Cells were treated with potential ligand source for 5 min at 37°C. Conditioned medium containing hPS and hGas6 was concentrated 15-fold and then diluted 3-fold and 30-fold to achieve the final concentrations, estimated to be 400 nM or 40 nM, respectively. Control-conditioned medium concentrated 15-fold was used at a 3-fold dilution for the studies. Cells were washed once with cold PBS, lysates were prepared, and receptors were immunoprecipitated with a polyclonal antibody that recognizes both hRse and mRse and were immunoblotted with an anti-phosphotyrosine antibody (4G10; UBI). After incubation with an horseradish peroxidase–conjugated anti-mouse IgG secondary antibody (Amersham), the phosphorylated proteins were revealed using a chemiluminescent detection method (ECL; Amersham).

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Stimulation of Sky Receptor Tyrosine Kinase by the Product of Growth Arrest-specific Gene *6**

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Kazumasa Ohashi‡, Kyoko Nagata‡, Jiro Toshima‡, Toru Nakano§, Hitoshi Arita§, Hiroko Tsuda¶, Koji Suzuki‖, and Kensaku Mizuno‡**

From the ‡Department of Biology, Faculty of Science, and the ¶Department of Clinical Chemistry and Laboratory Medicine, Faculty of Medicine, Kyushu University, Fukuoka 812, Japan, the §Shionogi Research Laboratories, Shionogi & Co., Ltd., Osaka 553, Japan, and the ‖Department of Molecular Pathobiology, Mie University School of Medicine, Mie 514, Japan

Sky (also called Rse, Brt, and Tyro3) is a member of a subfamily of related receptor tyrosine kinases, including Axl/Ufo/Ark and c-Eyk/Mer. We obtained evidence that Gas6 (the product of growth arrest-specific gene *6*) is a ligand of the Sky receptor tyrosine kinase. Gas6, but not protein S (an anticoagulant protein structurally similar to Gas6), specifically bound to the soluble form of Sky (Sky-Fc), composed of the extracellular domain of Sky fused to the Fc domain of human immunoglobulin G1. The native and recombinant Gas6, but not protein S, stimulated tyrosine phosphorylation of Sky ectopically expressed in Chinese hamster ovary cells. Stimulation of Sky in response to Gas6 was inhibited by Sky-Fc. The half-maximal concentration of Gas6 that stimulated Sky was about 1 nM. Thus, Gas6 as a ligand for Sky specifically binds to and stimulates Sky receptor tyrosine kinase.

Receptor tyrosine kinases play a central role in transducing the external signals across cell membranes into intracellular signaling systems and these signals lead to cell proliferation, differentiation, and other responses (1). Based on similarities of the sequences of kinase catalytic domains and the structural motifs in the extracellular domains, these receptors can be classified into subfamilies (1). The members of an Axl/Sky receptor subfamily, which include Axl (also called Ufo and Ark) (2–4), Sky (also called Rse, Brt, and Tyro3) (5–9), and c-Eyk (also called Mer) (10, 11), contain the characteristic extracellular ligand-binding domain composed of two immunoglobulin-like motifs and two fibronectin type III motifs. Axl was originally identified as the oncogene for human myelogenic leukemia (2, 3), and the gene for c-Eyk was isolated as a proto-oncogene for the avian viral oncogene v-*eyk* (10). Overexpression of Axl and Sky led to cell transformation (2, 9, 12). As the Axl/Sky family receptors have an oncogenic potential, they may be involved in tumor progression and in normal cell proliferation. Northern blot analysis revealed that the Sky transcripts are predominantly expressed in the brain (5–9), while those for Axl and c-Eyk are more widely distributed in various tissues (2–4, 10, 11).

The functional roles of the Axl/Sky subfamily of receptor tyrosine kinases have been given much attention, especially the identification of ligands. The ligands for Axl and Sky were recently reported to be the product of growth arrest-specific gene *6* (Gas6)[1] and protein S, respectively (13, 14). Protein S is a vitamin K-dependent plasma glycoprotein that has anticoagulant activity by acting as a cofactor of activated protein C (APC)-catalyzed inactivation of coagulation factors Va and VIIIa (15). Gas6, originally identified as a gene product expressed in response to growth arrest, has structural similarity to protein S with 42–43% identity (16) and was seen to function as a potentiating factor for thrombin-induced proliferation of vascular smooth muscle cells (VSMC) (17).

Prior to investigating the physiological functions of Sky and its reported ligand protein S, we examined the potential of protein S to bind to Sky and to stimulate tyrosine phosphorylation of Sky. In contrast to an earlier report (14), we found no specific binding of protein S to Sky or stimulation of Sky tyrosine phosphorylation by protein S. Gas6, not protein S, did show potent activity to bind to Sky and to induce the phosphorylation of Sky. We describe here our evidence that Gas6 is the ligand for Sky.

EXPERIMENTAL PROCEDURES

Purification of Gas6 and Protein S—Gas6 was purified from the conditioned medium of rat VSMC, according to the methods described elsewhere (17). The purity of Gas6 was confirmed by SDS-polyacrylamide gel electrophoresis (PAGE) analysis (17). Protein S was purified from normal human plasma by the reported procedures (18, 19). Based on the SDS-PAGE analysis, under reducing conditions, the preparations of protein S used in this study contained almost equal amounts of thrombin-cleaved and uncleaved protein S (18). The purified protein S had APC cofactor activity and also inhibited the activity of the platelet prothrombinase complex (20).

Expression of Recombinant Gas6 and Protein S—The cDNA encoding rat Gas6 was obtained as previously reported (17). The cDNA encoding human protein S was provided by Dr. B. Dahlbäck (21). The cDNAs were subcloned into the PUC-SRα expression plasmid (22), and the constructs were transfected into COS-7 cells using liposome methods (23). Cells were cultured for 3 days in serum-free Dulbecco's modified Eagle's medium with 10 μM sodium menadione bisulfite. Levels of proteins expressed in the conditioned media (100–1000 ng/ml) were determined by immunoblotting with anti-Gas6 or anti-protein S antibodies.

Antibodies—Rabbit anti-Sky polyclonal antibody (Sky-C) raised against the C-terminal peptide of human Sky was prepared and purified as described previously (24). Rabbit anti-protein S polyclonal antibody was raised against the purified human protein S, as described elsewhere (25). Rabbit anti-Gas6 antiserum was raised against the purified Gas6. Anti-phosphotyrosine monoclonal antibody (PY20) was purchased from ICN Biomedicals.

Construction and Purification of Fc Fusion Proteins—The cDNA encoding human Sky was isolated, as reported elsewhere (5). The cDNA encoding human c-Met (receptor for hepatocyte growth factor) was

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** To whom correspondence should be addressed. Fax: 81-92-632-2741.

[1] The abbreviations used are: Gas6, the protein encoded by growth arrest-specific gene *6*; APC, activated protein C; CHO, Chinese hamster ovary; EGF, epidermal growth factor; FGF, fibroblast growth factor; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline, VSMC, vascular smooth muscle cells.

obtained from Dr. G. F. Vande Woude (26). The cDNA for the Fc region of human IgG1 was amplified by polymerase chain reaction, using human placenta cDNA as a template (27). The *KpnI* fragment encoding the entire extracellular domain (amino acid residues 1–430) of the Sky protein was fused in-frame with the PCR-amplified cDNA fragment of the Fc region (residues 216–443) of human IgG1, resulting in the production of the cDNA coding for the Sky-Fc fusion protein with a Pro-Gly spacer sequence. In a similar manner, the cDNA for the extracellular domain (residues 1–941) of c-Met was ligated with the cDNA encoding the Fc region of human IgG1 to construct the cDNA for Met-Fc fusion protein with a spacer sequence, Pro-Gly-Val-Pro-Gly. The fused cDNAs were then subcloned into PUC-SRα expression vector (22) and transfected into COS-7 cells, using liposome methods. The serum-free conditioned media of COS cells were collected for three days. The Fc fusion proteins were purified by Protein A-Sepharose (Pharmacia Biotech Inc.) chromatography. Levels of the fusion proteins expressed in the conditioned media were on an average of 100–200 ng/ml.

Binding Assay of Gas6 and Protein S to Fc Fusion Proteins—Purified rat Gas6 or human protein S at a final concentration of 2 nM was incubated at 4 °C overnight with 5 nM Sky-Fc or Met-Fc fusion protein and Protein A-Sepharose (30 μl of 50% slurry) in 200 μl of Hanks' balanced salt solution containing 20 mM Hepes (pH 7.0), 0.02% NaN_3, and 1% bovine serum albumin. After centrifugation, the precipitates were washed four times with cold phosphate-buffered saline (PBS), suspended in SDS sampling buffer (25 mM Tris-HCl, pH 6.5, 5% glycerol, 1% SDS, 144 mM 2-mercaptoethanol, 0.05% bromphenol blue), and subjected to 8% SDS-PAGE. The gels were then transferred to polyvinylidene difluoride membrane (Bio-Rad) and the membrane was blocked with 3% nonfat dry milk in PBS containing 0.05% Tween 20 and incubated for 1 h at room temperature with anti-Gas6 or anti-protein S antibody diluted in PBS containing 1% nonfat dry milk and 0.05% Tween 20. After washing the membrane with PBS, it was incubated with the horseradish peroxidase-conjugated anti-rabbit IgG (Amersham Corp.) and immunoreactive bands were visualized using ECL chemiluminescence reagent (Amersham).

Construction of CHO Cell Lines Expressing Sky—A 3.8-kb human *sky* cDNA (5) was subcloned into the *NotI* site of the expression vector pRc/RSV (Invitrogen) containing the neomycin-resistant gene. The resulting plasmid was transfected into CHO cells using liposome methods, and the G418-resistant colonies were selected. One of the cell lines (B31) with high Sky expression, as measured by immunoblot analysis, was selected.

Tyrosine Phosphorylation Assay—B31 cells were plated on 60-mm dishes at a density of 2.5×10^4 cells/cm^2 and cultured in Ham's F-12 medium supplemented with 10% fetal calf serum. After 16 h, cells were serum-starved for 3 h and treated with Gas6 or protein S for 10 min at 37 °C, as indicated. Cells were rinsed once with cold PBS containing 1 mM orthovanadate and lysed with cold lysis buffer (20 mM Hepes, pH 7.2, 1% Nonidet P-40, 10% glycerol, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM orthovanadate, and 10 μg/ml leupeptin). The lysates were immunoprecipitated with anti-Sky antibody, run on SDS-PAGE, and immunoblotted with anti-Sky antibody or anti-phosphotyrosine monoclonal antibody, as described previously (24).

RESULTS AND DISCUSSION

To determine the binding potential of protein S and Gas6 to Sky, we prepared a chimeric protein (Sky-Fc) that contains the extracellular ligand-binding domain of Sky fused to the Fc region of human immunoglobulin IgG1 heavy chain. Affinity adsorption and precipitation analysis using Fc fusion proteins in the presence of Protein A-Sepharose showed that Sky-Fc, but not Met-Fc (a control chimeric protein composed of the extracellular domain of c-Met and the Fc region of human IgG1), specifically co-precipitated Gas6 protein (Fig. 1*A*). On the other hand, protein S was not co-precipitated by either Sky-Fc or Met-Fc (Fig. 1*B*). These observations suggest that Gas6 but not protein S can specifically bind to the extracellular domain of Sky.

Ligands for receptor tyrosine kinases bind to their cognate receptors, then rapidly stimulate tyrosine phosphorylation of these receptors. To determine if Gas6 and/or protein S could stimulate tyrosine phosphorylation of Sky, we constructed CHO cells stably expressing the full-length Sky receptor tyrosine kinase. Immunoblot analysis using the anti-Sky antibody

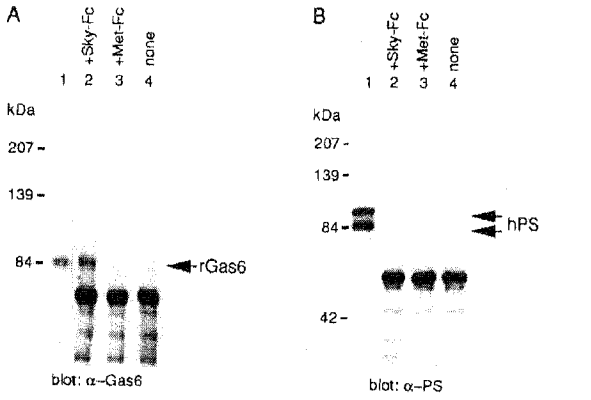

FIG. 1. **Binding of Gas6 to Sky-Fc fusion protein.** *A*, rat Gas6 (2 nM) purified from conditioned media of VSMC was incubated with Protein A-Sepharose in the absence (*lane 4*) or presence of 5 nM Sky-Fc (*lane 2*) or Met-Fc (*lane 3*) fusion protein. After centrifugation, the precipitates were run on SDS-PAGE and the bound Gas6 was visualized by immunoblotting with anti-Gas6 antibody (*α-Gas6*). *Lane 1* shows the immunoblot of the purified Gas6 used in this assay. *B*, protein S (2 nM) purified from human plasma was treated as in *A*, and the precipitates were immunoblotted with anti-protein S antibodies (*α-PS*). Elution positions of rat Gas6 (*rGas6*) and human protein S (*hPS*) are indicated by *arrows*. The upper and lower bands of protein S correspond to the intact and thrombin-cleaved form, respectively. Molecular sizes (kDa) of marker proteins are indicated on the *left*.

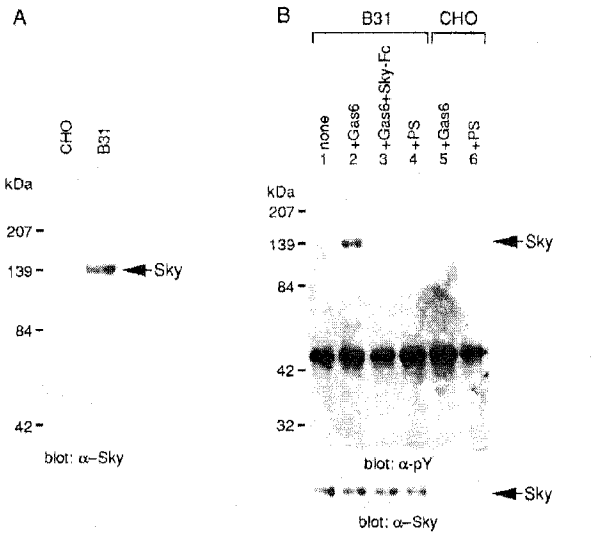

FIG. 2. **Tyrosine phosphorylation of Sky on B31 cells in response to purified Gas6.** *A*, immunoblot analysis of Sky expression on CHO cells and B31 cells. Cell lysates of parental CHO cells (*lane 1*) and *sky* cDNA-transfected CHO cells (B31 cells) (*lane 2*) were run on SDS-PAGE and immunoblotted with anti-Sky antibody (*α-Sky*). *B*, B31 cells (*lanes 1–4*) and CHO cells (*lanes 5* and *6*) were treated for 10 min at 37 °C with 2 nM Gas6 purified from conditioned media of VSMC or protein S (*PS*) purified from human plasma, as indicated. In *lane 3*, Gas6 (2 nM) was preincubated with excess amounts of Sky-Fc (20 nM). Cell lysates were immunoprecipitated with anti-Sky antibody, run on SDS-PAGE, and immunoblotted with anti-phosphotyrosine antibody (*α-pY, upper panel*) or anti-Sky antibody (*α-Sky, lower panel*). Elution position of Sky is indicated by an *arrow*. Molecular sizes (kDa) of marker proteins are indicated on the *left*.

raised against the C-terminal peptide of Sky revealed that the Sky protein with an apparent molecular mass of 140 kDa was detected in Sky-transfected (B31) cells, while the parental CHO cells showed no detectable immunoreactive band (Fig. 2*A*). Cell surface biotinylation experiments revealed that a 140-kDa protein immunoprecipitated with anti-Sky antibody was exposed at the cell surface (data not shown). The tyrosine

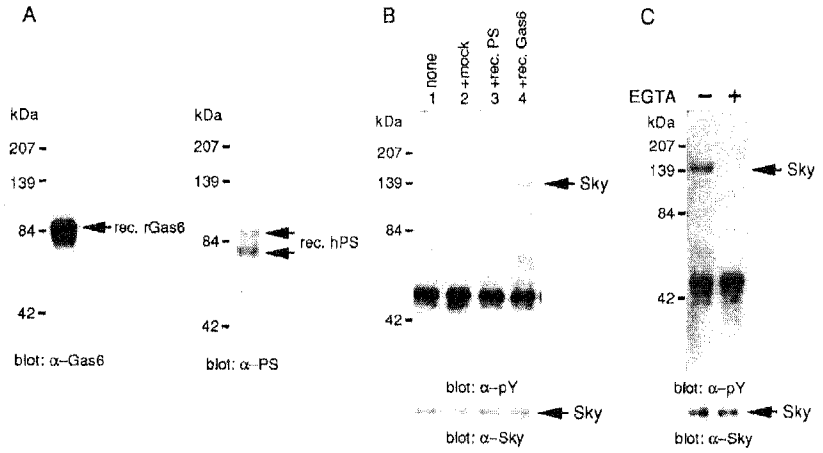

FIG. 3. **Tyrosine phosphorylation of Sky on B31 cells in response to recombinant Gas6.** *A*, immunoblot analysis of recombinant rat Gas6 and human protein S transiently expressed in COS-7 cells. The culture supernatants of COS cells transfected with rat Gas6 or human protein S cDNA expression plasmid were concentrated, run on SDS-PAGE, and immunoblotted with anti-Gas6 or anti-protein S antibody. *B*, B31 cells were treated for 10 min at 37 °C with the culture supernatants of mock-transfected COS cells (*lane 2*) or the cells transfected with human protein S (*lane 3*) or rat Gas6 expression plasmid (*lane 4*). *C*, effects of EGTA on tyrosine phosphorylation of Sky in response to Gas6. B31 cells were treated for 10 min at 37 °C with 2 nM recombinant rat Gas6 in the presence or absence of 10 mM EGTA. In *B* and *C*, cell lysates were immunoprecipitated with α-Sky, run on SDS-PAGE, and immunoblotted with α-pY (*upper panel*) or α-Sky antibody (*lower panel*), as described in Fig. 2. Elution position of Sky is indicated by an *arrow*. Molecular sizes (kDa) of marker proteins are indicated on the *left*.

phosphorylation of Sky expressed on B31 cells was assessed by immunoblotting with anti-phosphotyrosine antibody after immunoprecipitation of the cell lysates with anti-Sky antibody. As shown in Fig. 2*B*, phosphorylation of Sky was induced when the B31 cells were treated with Gas6 (compare *lanes 1* and *2*), but not when treated with protein S (*lane 4*), while the amount of Sky in the immunoprecipitates remained unchanged. No immunoreactive band with anti-phosphotyrosine antibody was detected in the anti-Sky immunoprecipitates of the parental CHO cells treated with Gas6 (Fig. 2*B*, *lane 5*). These results clearly show that Gas6 but not protein S can stimulate tyrosine phosphorylation of the Sky receptor tyrosine kinase expressed in B31 cells. Additionally, phosphorylation of Sky in response to Gas6 was almost completely blocked in the presence of excess amounts of Sky-Fc (Fig. 2*B*, *lane 3*). This means that Sky phosphorylation was induced by direct interaction between Gas6 and Sky and not by indirect cross-phosphorylation of Sky by other receptor tyrosine kinases.

To exclude the possibility that the Gas6 preparation purified from VSMC used in this study might be contaminated by the protein activating Sky, recombinant Gas6 was expressed in COS cells and examined to observe if it would stimulate Sky phosphorylation. Recombinant protein S was also expressed and examined, in parallel experiments. Expression of Gas6 and protein S in culture supernatants of COS cells transfected with each expression plasmid was confirmed by immunoblot analysis (Fig. 3*A*). As shown in Fig. 3*B*, the culture supernatants of COS cells expressing recombinant Gas6 induced tyrosine phosphorylation of Sky, whereas the supernatants of mock-transfected COS cells and the supernatants of COS cells expressing recombinant protein S had no detectable activity. These observations also show that Gas6 but not protein S stimulates Sky tyrosine phosphorylation.

Protein S contains 11 γ-carboxyglutamic acid (Gla) residues in the N-terminal Gla domain, one β-hydroxyaspartic acid residue in the first epidermal growth factor (EGF)-like domain, and three β-hydroxyasparagine residues in three other EGF-like domains (15). These modified residues appear to be involved in Ca^{2+} binding (15). As these residues are conserved in the sequence of Gas6, Gas6 may also bind Ca^{2+} ions through these residues (16, 17). To examine the effects of Ca^{2+} ion on Sky-stimulating activity of Gas6, recombinant Gas6 was treated with EGTA and lost all activity (Fig. 3*C*). Therefore, Ca^{2+} ion binding is essential for the conformation and activity of Gas6 to bind to and stimulate Sky.

Recombinant Gas6 purified to apparent homogeneity from the culture medium of COS cells transfected with Gas6 expression plasmid stimulated Sky phosphorylation in a dose-dependent manner (Fig. 4). Tyrosine phosphorylation of Sky was detectable at 0.5 nM of recombinant Gas6 and the half-maximal stimulation was obtained at approximately 1 nM. The concentration of Gas6 required for stimulation of Sky is comparable to the K_d value (0.3 nM) of the binding of Gas6 to membranes of VSMC, for which Gas6 has growth potentiating activity (17).

In light of all these findings, we propose that Gas6, but not protein S, is a ligand for Sky, specifically binding to Sky and stimulating tyrosine phosphorylation. Our observations differ from those of Stitt *et al.* (14). They reported that protein S but not Gas6 efficiently binds to and stimulates mouse Sky (Tyro3). Although the Sky receptor they used was of a different species (mouse Sky used by Stitt *et al.* (14) *versus* human Sky used in our study), the species difference would not likely explain the different results in ligand specificity, because sequences of human and mouse Sky are highly homologous (85% identity within their extracellular domains). If all results are compiled, human protein S does bind to mouse Sky (as described by Stitt *et al.*), but does not bind to human Sky (as described here). As the purified human protein S used in our study retained both APC cofactor activity and inhibitory activity of the prothrombinase complex activity (20), it is unlikely that the purified protein S we used was structurally damaged. Thus, at present we have no valid explanation for the discrepancy between the results obtained in this study and those by Stitt *et al.* (14).

Two different research groups reported that Gas6 is the ligand for Axl, a receptor closely related to Sky (13, 14). The effective dose of Gas6 to Sky shown in this study is comparable to the reported value for Gas6-Axl interaction (13). Thus, Gas6 may be a common ligand for the two related receptors, Sky and Axl. The binding of a ligand to two distinct members of a receptor subfamily is also seen for fibroblast growth factor (FGF) family ligands (acidic FGF and basic FGF), both of which bind to two members of an FGF receptor subfamily, Flg and Bek, with similar affinity constants (28), and the ligands for Eph family receptors (B61 and EHK1-L), which bind to two

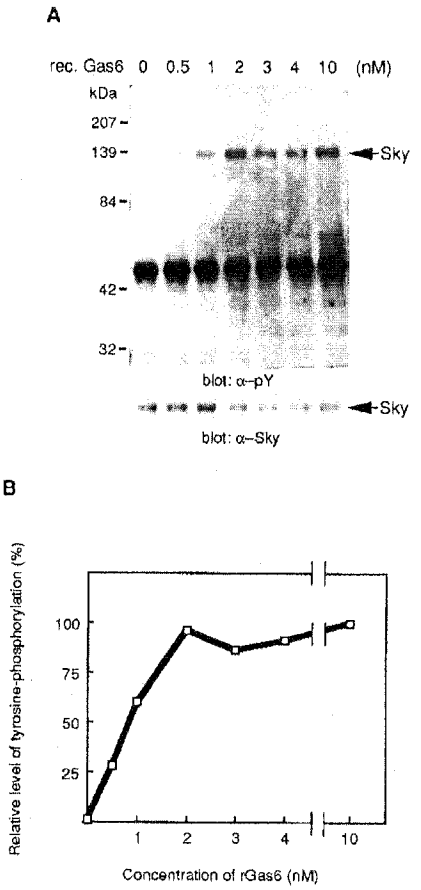

FIG. 4. **Dose-dependent tyrosine phosphorylation of Sky in response to purified recombinant rat Gas6.** Recombinant Gas6 was purified from the culture supernatants of COS-7 cells transfected with rat Gas6 expression plasmid. Serum-starved B31 cells were treated for 10 min at 37 °C with the indicated concentrations of purified recombinant Gas6. Cell lysates were immunoprecipitated and immunoblotted with α-pY (*upper panel*) or α-Sky antibody (*lower panel*), as described in Fig. 2. Elution position of Sky is indicated by an *arrow*. Molecular sizes (kDa) of marker proteins are indicated on the *left*. *B*, relative levels of tyrosine phosphorylation of Sky are plotted as a function of the concentration of Gas6 added. The levels of tyrosine phosphorylation of Sky were calculated by dividing the density of anti-phosphotyrosine immunoreactive band by the density of anti-Sky immunoreactive band. The density was evaluated from data in *A* by NIH image software. The level of tyrosine phosphorylation of Sky when treated with 10 nM recombinant rat Gas6 was taken as 100%.

members of an Eph receptor subfamily, Eck and EHK-1, with similar affinity constants (29). Although neither the functions of Gas6 nor the physiological significance of receptor redundancy are well understood, Gas6 may exhibit diverse functions in a cell and tissue-dependent manner through two distinct receptors, Sky and Axl, whose expression patterns differ significantly.

Identification of the ligand for Sky should pave the way to initiate research on the functional roles of Sky and its ligand Gas6. As Sky is expressed predominantly in neurons in restricted regions of the brain (6), future study will focus on the biological function of Gas6 on neurons. As Sky is also expressed in some extents in other tissues, such as testis, ovary and kidney, and in certain types of cells (5–9, 12), the functional roles of Gas6 to these tissues and cells may be identified. Gas6 potentiates cell proliferation of VSMC stimulated by Ca^{2+}-mobilizing growth factors, such as thrombin, and may be involved in intimal thickening of the vascular wall accompanying atherosclerosis or restenosis (17). To develop antagonists for Gas6 that will aid in overcoming these vascular diseases, it is also important to clearly define which receptor, Sky or Axl, mediates the action of Gas6 on VSMC. In addition, it will be interesting to search for other members of the protein S-related protein family, which may function as ligands for Sky-related receptors such as c-Eyk.

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Hemostasis, thrombosis models and thrombolysis. We anesthetized mice by intraperitoneal injection of sodium pentobarbital (60 mg/kg). Thrombus formation due to stasis was induced by tightening two sutures, separated 0.7 cm apart in the inferior *vena cava*, for 20 min[35]. Thrombosis was quantified by weighing the thrombus after rinsing, blotting on filter paper and drying overnight at 60 °C (ref. 35). For rescue experiments, *Gas6*$^{-/-}$ mice received 100 µg/kg of r*Gas6*. Thrombus formation in the carotid artery by photochemical denudation of the endothelium was established by irradation of the exposed artery with green light (wavelength: 540 nm) from a xenon lamp (L4887, Hamamatsu Photonics, Hamamatsu, Japan) after intravenous administration of Rose Bengal as described[37]. To induce thrombo embolism, a mixture of collagen (0.5 mg/kg, equine collagen; Hormon Chemie, München, Germany) and epinephrine (60 µg/kg) was injected into the jugular vein[28]. When indicated, mice received 100 µg goat-anti-human Gas6 (directed against the C-terminal part of Gas6) or control isotype-matched antibodies (Santa Cruz Biotechnology, Santa Cruz, California). Thrombolysis of PRP or *P* plasma clots embolized into the lungs was measured as described[38]. Bleeding was measured by tail tip transsection as described[39].

Platelet aggregation and secretion. Whole blood, drawn from anesthetized mice from the inferior *vena cava* into 4% citrate (1 volume anticoagulant/9 volumes blood), was centrifuged at 100*g* (10 min) to obtain PRP and additionally at 2,000*g* (10 min) to obtain *P*. PRP and *P* were pooled from four *Gas6*$^{-/-}$ or wild-type mice. Washed platelets were prepared with blood drawn from the inferior *vena cava* into acid-citrate-dextrose solution (ACD) (1 volume ACD /6 volumes blood). Apyrase was added to PRP (final concentration, 1 U/ml), and platelets were washed by adding 2 vol ACD and centrifuged at 2,000*g* (10 min). The platelet pellet was resuspended in Tyrode's buffer containing 1% BSA. For experiments using washed human platelets, blood from volunteers (9 volumes) was anticoagulated with 3.13% citrate (1 volume). Washed platelets were prepared as mentioned above.

We measured platelet aggregation turbidimetrically using an optical Chronolog aggregometer (model 490, Coulter, Hialeah, Florida). When indicated, human washed platelets were preincubated with anti-Gas6 or irrelevant antibodies (Santa Cruz) before stimulation with ADP (4 min). Platelet ATP release was monitored by adding firefly luciferase and luciferin and comparing the luminescence generated by platelet ATP release or an ATP standard (Chrono-Lume, Kordia, The Netherlands). When indicated, platelet aggregation or ATP release were performed in the presence of recombinant murine Gas6. TXA_2 production was measured by TXB_2 assay using an EIA Biotrak kit (Amersham International, UK).

Production of rGas6, western-blot analysis and PCR analysis. rGas6 was produced in 293 cells stably transfected with the pcDNA3 expression vector encoding the mouse *Gas6* cDNA. rGas6 was purified using a modification of a calcium affinity method used for Gla-containing proteins[40,41]. Washed platelet extracts were immunoblotted after separation by SDS-PAGE using the following antibodies: anti-human Gas6 and anti-human Axl (both from Santa Cruz), anti-murine Sky or anti-murine fibrinogen (Nordic Immunology, Tilburg, The Netherlands). PCR was performed on a human platelet cDNA library as described[42] using the following primers: for *Gas6*, 5´-AGCTGCTCGAGGCGCTGTTGCCGGCGC-3´ and 5´-AGCTGCTCGAGGACCAGTGCACGCCCAACC-3´ (ref. 27); for *axl*, 5´-GGTGGCTGTGAAGACG ATGA-3´ and 5´-CTCAGATACTCCATGCCATCT-3´ (ref. 29); for *mer*, 5´-CACCTCTGCCTTACCACAT CT-3´ and 5´-ATCCACAAAAGCAGCCCAA AGA-3´ (ref. 43); for *sky*, 5´-CAATCTGAGCACGCTACCAA-3´ and 5´-GGACAGAAGAGGCTGTCCAG-3´.

Electron microscopy and flow cytometry. Washed platelets, stimulated by thrombin 1 U/ml (5 min) under stirring conditions, were fixed with 2% glutaraldehyde in sodium cacodylate-HCl buffer (pH 7.2-7.4) and post-fixed in 1% osmium tetraoxide in the same buffer before embedding in Epon resin. For immuno EM, 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 was used as described[44]. The sections were examined using a Philips 201 electron microscope. To analyze surface expression of Gas6, washed resting and stimulated platelets were incubated with goat anti-human Gas6 antibody (Santa Cruz) and, subsequently, with fluorescein isothiocyanate (FITC)-rabbit anti-goat antibody (Dako, Glostrup, Denmark). For surface expression of fibrinogen or P-selectin, a FITC-fibrinogen rabbit polyclonal antibody (Dako, Glostrup, Denmark) or a FITC-P-selectin rat anti-mouse antibody (BD PharMingen, San Diego, California) were used. After incubation, samples were diluted and immediately analyzed on a FACS calibur flow cytometer (Becton Dickinson, Rungis, France).

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Deficiency or inhibition of Gas6 causes platelet dysfunction and protects mice against thrombosis

ANNE ANGELILLO-SCHERRER[1], PABLO GARCIA DE FRUTOS[2], CRISTINA APARICIO[2], ELS MELIS[1], PIERRE SAVI[3], FLOREA LUPU[4], JEF ARNOUT[1], MIEKE DEWERCHIN[1], MARC F. HOYLAERTS[1], JEAN-MARC HERBERT[3], DÉSIRÉ COLLEN[1], BJÖRN DAHLBÄCK[2] & PETER CARMELIET[1]

[1]*The Center for Transgene Technology and Gene Therapy, Flanders Interuniversity Institute for Biotechnology, Leuven, Belgium*
[2]*Department of Clinical Chemistry, Lund University, Wallenberg Laboratory, Malmö University Hospital, Malmö, Sweden*
[3]*Cardiovascular/Thrombosis Research Department, Sanofi–Synthélabo, Toulouse, France*
[4]*Thrombosis Research Institute, Vascular Biology Laboratory, London, UK*
A.A.S. and P.G.F. contributed equally to this study.
Correspondence should be addressed to P.C.; email: peter.carmeliet@med.kuleuven.ac.be

The growth arrest-specific gene 6 product (Gas6) is a secreted protein related to the anticoagulant protein S but its role in hemostasis is unknown. Here we show that inactivation of the *Gas6* gene prevented venous and arterial thrombosis in mice, and protected against fatal collagen/epinephrine-induced thrombo embolism. *Gas6*$^{-/-}$ mice did not, however, suffer spontaneous bleeding and had normal bleeding after tail clipping. In addition, we found that Gas6 antibodies inhibited platelet aggregation *in vitro* and protected mice against fatal thrombo embolism without causing bleeding *in vivo*. Gas6 amplified platelet aggregation and secretion in response to known agonists. Platelet dysfunction in *Gas6*$^{-/-}$ mice resembled that of patients with platelet signaling transduction defects. Thus, Gas6 is a platelet-response amplifier that plays a significant role in thrombosis. These findings warrant further evaluation of the possible therapeutic use of Gas6 inhibition for prevention of thrombosis.

Gas6, the product of the growth arrest-specific gene 6 (Gas6), is a new member of the vitamin K-dependent protein family[1,2]. Proteins belonging to this family are characterized by post-translational γ-carboxylation of certain glutamic acid residues by a carboxylase, using vitamin K as cofactor. The γ-carboxyglutamic acid (Gla)-containing module in prothrombin, coagulation factors VII, IX and X, protein C, protein Z, protein S and Gas6 allows these vitamin K-dependent plasma proteins to bind to negatively charged phospholipid membranes[3]. Gas6 is structurally similar to protein S, but lacks a loop, crucial for the anticoagulant activity of protein S (ref. 2). The latter is a cofactor for activated protein C, which inactivates the coagulation factors Va and VIIIa (ref. 4). Genetic deficiency of protein S in humans is one of the most severe inherited risk factors for thrombosis[5]. To date, Gas6 is the only protein among Gla-module-containing proteins which has not been reported to play a role in hemostasis or thrombosis.

Apart from a Gla-domain-dependent interaction with phospholipid membranes[6], Gas6 also binds as a ligand to the receptor tyrosine kinases Axl (Ark, Ufo, Tyro7), Sky (Rse, Tyro3, Dtk, Etk, Brt, Tif) and Mer (c-Mer, Eyk, Nyk)[7–11] by its carboxy-terminal globular G domains[9]. It has been implicated in reversible cell growth arrest[2], survival[12], proliferation[12–14] and cell adhesion[6,15,16]. Mice with a triple deficiency of Axl, Sky and Mer are viable, but have not been reported to suffer spontaneous bleeding or thrombosis[17].

Here, we generated *Gas6*$^{-/-}$ mice to investigate the role of Gas6 in hemostasis and thrombosis. We found that deficiency of Gas6 protected mice against fatal thrombosis, but did not induce bleeding. Gas6 was found to amplify the aggregation and secretion response of platelets to known agonists, and the platelet dysfunction in Gas6-deficient mice resembled the platelet defects of patients with primary platelet signal transduction defects[18]. Gas6 antibodies protected mice against fatal thrombo embolism without inducing a bleeding tendency, indicating that inhibition of Gas6 might provide a novel means to safely block thrombosis.

Results

Normal hemostasis in *Gas6*$^{-/-}$ mice

We inactivated *Gas6* by deleting the transcription start site, the translation initiation codon, the signal peptide and the Gla module that is required for binding of Gas6 to phospholipid membranes. We confirmed correct targeting at the DNA, RNA and protein level (Fig. 1*a–c*; Fig. 5*b*). Homozygous *Gas6*$^{-/-}$ mice were born at the expected mendelian frequency: of 317 offspring from heterozygous *Gas6*$^{+/-}$ breeding pairs, 72 were wild-type (*Gas6*$^{+/+}$), 168 were *Gas6*$^{+/-}$ and 77 were *Gas6*$^{-/-}$. *Gas6*$^{+/-}$ and *Gas6*$^{-/-}$ mice were viable, fertile, appeared normal and showed no obvious differences in size, weight or behavior. No genotypic differences in litter size were observed (9.7 ± 2 for wild-type mice; n = 23 litters versus 9.3 ± 3 for *Gas6*$^{-/-}$ mice; n = 44 litters; P = n.s.).

Gas6$^{-/-}$ mice did not suffer spontaneous bleeding or thrombosis. Bleeding (estimated as the amount of blood loss) after tail clipping was comparable for both genotypes (166 ± 48 μl in wild-type mice versus 172 ± 68 μl in *Gas6*$^{-/-}$ mice; n = 10; P = n.s.). There were also no genotypic differences in the plasma levels of

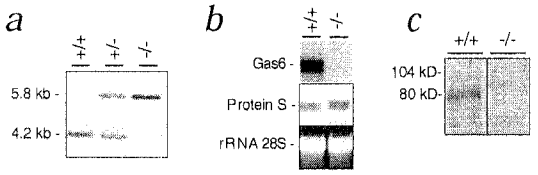


Fig. 1 Targeting of the *Gas6* gene. ***a***, Southern blot of HindIII-digested genomic DNA hybridized with an internal probe (within the 5′-flank), generating a 4.2 kb wild-type and a 5.8 kb *Gas6*-null fragment. ***b***, Northern-blot analysis of total RNA from cultured mouse embryonic fibroblasts, hybridized with a *Gas6* or *protein S* cDNA probe; ethidium bromide stained rRNA bands indicate equal loading. ***c***, Immunoblot analysis of kidney extracts using a rabbit polyclonal antibody against the murine C-terminal Gas6 peptide.

coagulation factors (percent of wild-type: 88 ± 13, 110 ± 8, 94 ± 9, 111 ± 21, 93 ± 8 and 90 ± 5 for fibrinogen, factor II, V, VIII, IX and X; n = 7–12 mice; P = n.s.), the prothrombin and activated partial thromboplastin (aPTT) times (percent of wild-type: 100 ± 5 and 85 ± 25, respectively; P = n.s.), and the counts of peripheral red cells, leukocytes and platelets (percent of wild-type: 102 ± 5, 89 ± 14 and 98 ± 5, respectively; n = 7–12; P = n.s.).

Protection of *Gas6*$^{-/-}$ mice against thrombosis

We used three thrombosis models to determine the effects of Gas6 deficiency. In the first model, thrombosis was induced by ligation of the abdominal caval vein. Stasis-induced venous thrombosis is known to result from both coagulation and platelet activation[19]. Compared with wild-type mice, thrombi in *Gas6*$^{-/-}$ mice were on average 85% smaller (P < 0.001; Fig. 2*a*). In the second model, we induced a platelet- and fibrin-rich thrombus by photochemical denudation of the carotid artery[20]. Compared with wild-type mice, the arterial thrombus in *Gas6*$^{-/-}$ mice was on average 60% smaller (P < 0.05; Fig. 2*b*). In the third model, where we induced platelet-dependent thrombo embolism by intravenous injection of collagen and epinephrine in anesthetized mice, 80% of wild-type mice died within 1 to 3 minutes as compared with 20% of *Gas6*$^{-/-}$ mice that died within 10 to 15 minutes (n = 10; P < 0.03; Fig. 2*c*). These data indicate that thrombus formation in *Gas6*$^{-/-}$ mice did not develop as rapidly and extensively as in wild-type mice. Macroscopic and histological analysis revealed extensive pulmonary thrombo embolism in wild-type mice (Fig. 2*e*). In contrast, surviving *Gas6*$^{-/-}$ mice lacked signs of pulmonary embolization (Fig. 2*f*). Thus, loss of Gas6 significantly protected mice against venous and arterial thrombosis.

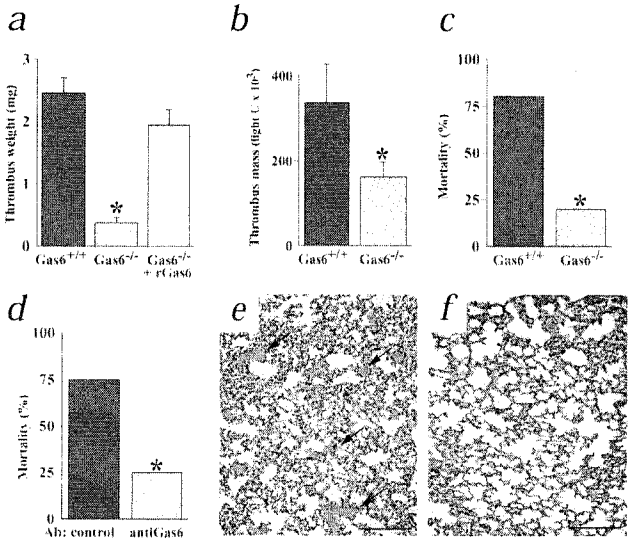


Fig. 2 Inactivation or inhibition of Gas6 protects mice against thrombosis. ***a***, Stasis-induced thrombosis in the inferior *vena cava* (n = 10; mean ± s.e.m.; *, P < 0.001); recombinant murine Gas6 restored thrombosis in *Gas6*$^{-/-}$ mice (□). ***b***, Thrombosis in the carotid artery induced by endothelial denudation (n = 5; *, P < 0.05). ***c*** and ***d***, Thrombo embolism induced by collagen/epinephrine injection in both genotypes (*c*; n = 10) and in wild-type mice injected with anti-Gas6 antibodies or control antibodies (*d*; , n=12; ■, n = 16; *, P < 0.03). ***e*** and ***f***, Light microscopy (H&E staining) of the lungs after collagen/epinephrine injection, revealing extensive platelet thrombo embolism (arrows) in wild-type mice (*e*) but not in surviving *Gas6*$^{-/-}$ mice (*f*). Scale bars, 400 μm.

The resistance to thrombosis of *Gas6*$^{-/-}$ mice was not due to increased thrombolysis. Lysis of [^{125}I]fibrin-labeled pulmonary plasma clots was comparable in both genotypes, regardless of whether platelet-poor (*P*) or platelet-rich plasma (PRP) was used to make these clots. Lysis of PRP-clots after 16 hours was 33 ± 10% in wild-type mice versus 30 ± 7% in *Gas6*$^{-/-}$ mice (n = 6; P = n.s.), while lysis of *P*-clots after 16 hours was 67 ± 8% in wild-type mice versus 52 ± 5% in *Gas6*$^{-/-}$ mice (n = 6; P = n.s.).

Impaired platelet aggregation in *Gas6*$^{-/-}$ mice

The prothrombotic mechanisms of Gas6 were further studied by analyzing its effects on blood coagulation and fibrin formation. Recombinant murine Gas6 protein (rGas6; 0.2–1 μg/ml) did not affect the aPTT of *Gas6*$^{-/-}$ plasma (in seconds: 33 ± 2, 34 ± 1, 35 ± 1 and 34 ± 3 at 0, 0.2, 0.4 and 1 μg/ml rGas6; n = 6; P = n.s.), consistent with previous findings in human plasma that rGas6 only minimally altered the aPTT or the degradation of factor V induced by activated protein C (ref. 21).

As platelets are known to play an essential part in the venous and arterial thrombosis models used to demonstrate the antithrombotic *Gas6*$^{-/-}$ phenotype, we examined the role of Gas6 in platelet function. *Gas6*$^{-/-}$ mice had a normal platelet count and number and morphology of megakaryocytes (data not shown). In addition, *Gas6*$^{-/-}$ platelets appeared ultrastructurally normal (data not shown). However, platelet aggregation studies revealed significant functional defects in *Gas6*$^{-/-}$ mice. Platelets from wild-type mice dose-dependently aggregated in response to ADP (Fig. 3*a–d*), collagen (Fig. 3*e–h*) or the TXA_2 analogue U46619 (Fig. 3*i* and *j*). We achieved maximal aggregation of platelets from wild-type mice at similar concentrations of these agonists as used previously[22]. In contrast, platelets from *Gas6*$^{-/-}$ mice failed to irreversibly aggregate in response to low concentrations of ADP (< 10 μM), collagen (2 μg/ml) or U46619 (10 μM). At low agonist concentration, *Gas6*$^{-/-}$ platelets only displayed shape change as revealed by an immediate decrease in light transmission after stimulation. However, higher concentrations of ADP (50 μM; Fig. 3*d*), collagen (5–15 μg/ml; Fig. 3*f–h*) or U46619 (100 μM; Fig. 3*j*) induced irreversible aggregation of *Gas6*$^{-/-}$ platelets. Both wild-type and *Gas6*$^{-/-}$ platelets aggregated normally in response to the phorbol ester phorbol-12-myristyl-13-acetate (PMA) or the Ca^{++}

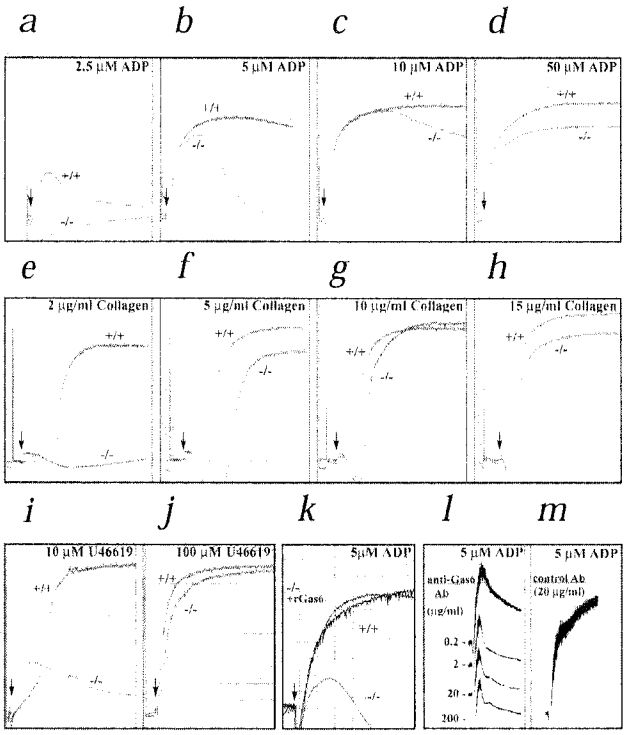


Fig. 3 Effect of Gas6 deficiency or of anti-Gas6 antibodies on platelet aggregation. ***a–j***, Aggregation of wild-type (+/+) and Gas6 deficient (–/–) platelet-rich plasma. Platelets from *Gas6*$^{-/-}$ mice were unable to fully aggregate in response to concentrations of ADP at 2.5 µM (*a*), 5 µM (*b*) and 10 µM (*c*), of collagen at 2 µg/ml (*e*) or of the thromboxane A_2 analogue U46619 at 10 µM (*i*), while higher concentrations of ADP (*d*; 50 µM), collagen (*f–h*; 5–15 µg/ml) or U46619 (*j*; 100 µM) induced irreversible platelet aggregation. Representative example of 4 independent experiments using PRP pooled from 4 to 6 wild-type or *Gas6*$^{-/-}$ mice. Squares represent 2 min (X-axis) and 10% change in light transmission (Y-axis). ***k***, Restoration of the impaired aggregation of *Gas6*$^{-/-}$ platelets in response to ADP (5 µM) by recombinant Gas6 (1 µg/ml) to comparable levels as in wild-type platelets. Representative example of 3 independent experiments. ***l*** and ***m***, Aggregation response to ADP (5 µM) of washed human platelets after preincubation with anti-Gas6 antibodies (*l*) or isotype-matched control antibodies (*m*), revealing that anti-Gas6 antibodies prevent platelet aggregation. Representative example of 3 independent experiments. Arrows in all panels indicate application of the platelet agonists.

ionophore A23187 (data not shown).

Thrombin stimulated platelet aggregation comparably in both genotypes at all doses tested (data not shown). However, ultrastructural analysis revealed that thrombin-induced aggregates of *Gas6*$^{-/-}$ platelets were abnormal. In wild-type aggregates, platelets were densely packed, made tight contacts with each other and were completely degranulated (Fig. 4*a*). In contrast, platelets in *Gas6*$^{-/-}$ aggregates were loosely packed, displayed fewer and smaller contact sites and were incompletely degranulated (Fig. 4*b*). Fibrinogen is released from α-granules upon platelet activation and forms bridges, linking adjacent activated platelets[23]. Flow cytometry of washed platelets revealed that fibrinogen was detectable on the surface of wild-type platelets after stimulation with ADP (20 µM; Fig. 4*c*). In contrast, levels of surface-bound fibrinogen in *Gas6*$^{-/-}$ platelets did not increase upon stimulation (Fig. 4*d*), which may contribute to the loose assembly of *Gas6*$^{-/-}$ platelet aggregates. The reduced amount of fibrinogen on *Gas6*$^{-/-}$ platelet surfaces was not due to a defect of *Gas6*$^{-/-}$ platelets to sequester fibrinogen, as revealed by the comparable amounts of immunoreactive fibrinogen in platelet lysates in both genotypes (Fig. 4*e*). Thus, impaired platelet aggregation in response to known agonists in *Gas6*$^{-/-}$ mice contributed to their resistance to thrombosis.

Expression of Gas6 and its receptors in platelets

The defect of platelet aggregation in *Gas6*$^{-/-}$ mice indicated that platelets produce and respond to Gas6. Therefore, the expression of Gas6 and its receptors Axl, Sky and Mer was studied in resting and stimulated platelets. By reverse transcriptase (RT)-PCR analysis, *GAS6* mRNA transcripts were detected in human platelets (Fig. 5*a*). In addition, immunoblotting revealed the presence of Gas6 in platelet extracts and in the releasate of thrombin-activated platelets from wild-type but not from *Gas6*$^{-/-}$ mice (Fig. 5*b*). Ultrastructural analysis combined with double immunogold-labeling of resting wild-type platelets revealed that Gas6 colocalized with fibrinogen in α-granules (data not shown). Upon activation of wild-type platelets with thrombin (1 U/ml), Gas6 became detectable on the surface of platelets by immunogold-labeling (data not shown). Flow cytometry confirmed that the levels of surface-bound Gas6 were minimal in resting human platelets but significantly increased upon activation by ADP (5 µM; Fig. 5*c* and *d*). These results extend previous observations of Gas6 in human megakaryocytes[24] and rat platelets[25].

Platelets also expressed Gas6 receptors. By RT-PCR analysis, transcripts of Axl, Sky and Mer were detected in human platelets (Fig. 5*a*). Immunogold-labeling revealed that Axl was localized on the surface of resting platelets (data not shown). Comparable amounts of Axl and Sky (Fig. 5*b*) were detectable in wild-type and *Gas6*$^{-/-}$ platelets, indicating that the reduced response of *Gas6*$^{-/-}$ platelets was not due to differences in Gas6 receptor expression. Thus, platelets produce and release Gas6, and express Gas6 receptors.

Impaired secretion in *Gas6*$^{-/-}$ platelets

Ultrastructural analysis of the thrombin-induced platelet aggregates indicated a reduced ability of *Gas6*$^{-/-}$ platelets to degranulate. Secretion of ADP from dense granules is essential for the formation of stable macro-aggregates after initial formation of small, unstable platelet aggregates[26]. Secretion of dense granule

Table 1 ATP release in response to various agonists.

Agonist	Concentration	Wild-type mice	*Gas6*$^{-/-}$ mice
ADP	20 µM	0.8 ± 0.08	0.2 + 0.09*
	20 µM + r*Gas6*	n.d.	0.75 ± 0.1
	50 µM	2.6 ± 0.8	1.3 ± 0.3*
Collagen	1 µg/ml	1.8 ± 0.3	< 0.1
	10 µg/ml	10 ± 1.0	11 ± 0.8
U46619	10 µM	6.5 ± 1.5	< 0.1
	100 µM	8.2 ± 2.0	7.6 ± 2.3
Thrombin	1 U/ml	17 ± 1.6	11 ± 1.2*
PMA	100 µM	2.3 ± 0.4	1.9 ± 0.6
A23187	8 µM	9.2 ± 3.1	7.3 ± 3.0

ATP release expressed in µM. The data represent the mean ± s.e.m. of 3 experiments using platelet-rich plasma (for ADP, collagen, U46619, PMA or A23187 stimulation) or washed platelets (for thrombin stimulation). Note that rGas6 (200 ng/ml) rescued the impaired ATP secretion of *Gas6*$^{-/-}$ platelets. Each experiment was performed with a pool of 4 to 6 wild-type or *Gas6*$^{-/-}$ mice. n.d. not done. *, $P < 0.05$ versus wild-type.

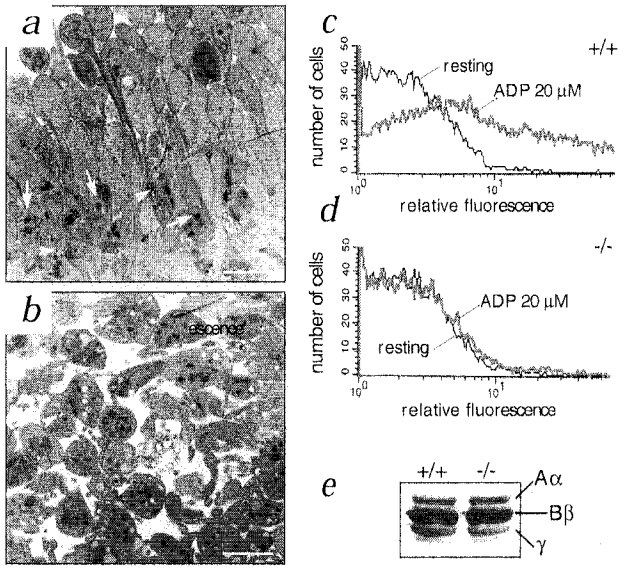
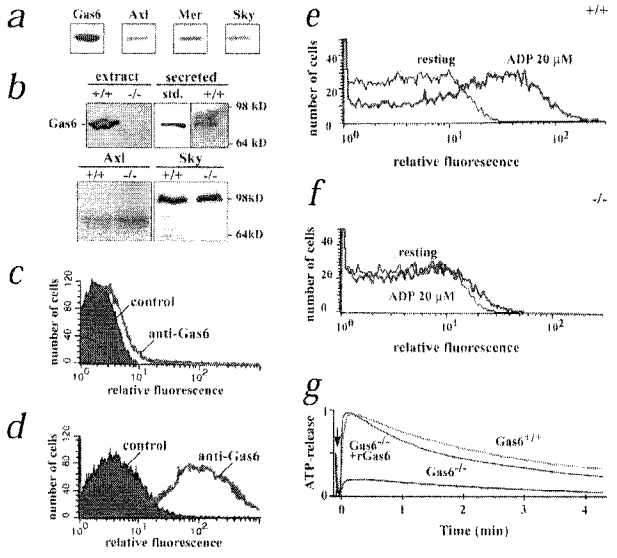


Fig. 4 Role of Gas6 in the formation of platelet macro-aggregates. ***a*** and ***b***, Electron microscopy revealed that platelets at the borders of platelet aggregates in wild-type mice (+/+) were densely compacted and completely degranulated (*a*). In contrast, Gas6 deficient (-/-) platelet aggregates of a comparable size were loosely packed, had fewer contact sites and were incompletely degranulated (*b*). Arrows indicate α-granules and arrowheads indicate dense granules in *a* and *b*. Scale bars, 4 µm. ***c*** and ***d***, Flow-cytometry analysis of fibrinogen on washed wild-type (*c*) and *Gas6*$^{-/-}$ (*d*) platelets (black line denotes resting platelets, green line denotes ADP-activated platelets), revealing that surface-bound fibrinogen levels only increased in stimulated wild-type but not in *Gas6*$^{-/-}$ platelets. ***e***, Western-blot analysis of washed platelet lysates reveals comparable amounts of fibrinogen in wild-type and *Gas6*$^{-/-}$ platelets.

stores (evaluated by measuring release of ATP) was significantly impaired in *Gas6*$^{-/-}$ platelets. Compared with wild-type platelets, release of ATP from *Gas6*$^{-/-}$ platelets was significantly decreased in response to ADP, collagen or U46619, when these agonists were used at low concentrations, which only caused platelet shape changes or reversible platelet aggregation (Table 1). ATP release from *Gas6*$^{-/-}$ platelets was also reduced in response to high concentrations of ADP (50 µM) or thrombin (1 U/ml), consistent with the incomplete degranulation of thrombin-stimulated *Gas6*$^{-/-}$ platelets (ultrastructural analysis). However, release of ATP was normal or only slightly reduced when *Gas6*$^{-/-}$ platelets were stimulated with high concentrations of collagen (10 µg/ml) or U46619 (100 µM; Table 1), which cause irreversible platelet aggregation. PMA and the Ca^{++} ionophore A23187 induced a normal secretory response in both genotypes (Table 1). Secretion of α-granules, as assessed by measurement of surface expression of P-selectin during platelet activation, was also impaired in *Gas6*$^{-/-}$ platelets (Fig. 5*e* and *f*). Thus, we found a close correlation between the defects in the aggregation and secretion response of *Gas6*$^{-/-}$ platelets to various agonists.

Production of TXA_2, which contributes to the formation of stable macro-aggregates[26], was normal in both genotypes. Production of TXA_2 in serum and upon activation of platelets by thrombin (5 U/ml) was estimated by measurement of TXB_2. Levels of TXB_2 were 110 ± 63 ng/ml in serum and 53 ± 23 ng/ml after thrombin activation of wild-type mice versus 120 ± 41 ng/ml in serum and 55 ± 16 ng/ml after thrombin activation of *Gas6*$^{-/-}$ mice (n = 6; P = n.s.).



Restoration of the *Gas6*$^{-/-}$ phenotype by recombinant *Gas6*

To confirm that the platelet dysfunction in *Gas6*$^{-/-}$ mice was due to deficiency of Gas6, we evaluated the effect of recombinant murine *Gas6* (rGas6) on the impaired aggregation and secretion of *Gas6*$^{-/-}$ platelets *in vitro*. Whereas rGas6 itself—at a concentration of up to 10 µg/ml—was unable to induce a shape change or aggregation of wild-type or *Gas6*$^{-/-}$ platelets, a concentration of 200 ng/ml restored the defective aggregation (Fig. 3*k*) and ATP secretion (Fig. 5*g*) of *Gas6*$^{-/-}$ platelets in response to ADP. Moreover, the thrombotic defect in *Gas6*$^{-/-}$ mice *in vivo* was restored by administering rGas6 at a dose of 100 µg/kg (n = 8; P > 0.05 by comparison to wild-type mice; Fig. 2*a*). These findings indicate that Gas6 stimulates platelet function by amplifying the response to other platelet activators.

Antibodies against Gas6 inhibit platelet function

In order to examine whether inhibitors of Gas6 might be useful to prevent thrombosis, we studied the effect of antibodies specific for Gas6 on platelet aggregation *in vitro* and on thrombo

Fig. 5 Expression and role of Gas6 in platelets. ***a***, RT-PCR analysis of Gas6 and its receptors Axl, Sky and Mer in human platelets. ***b***, Western-blot analysis revealing Gas6 in extracts of resting platelets and in releasates of thrombin-activated platelets from wild-type but not from *Gas6*$^{-/-}$ mice and comparable expression of the Gas6 receptors Axl and Sky. ***c*** and ***d***, Flow cytometry using Gas6-antibodies, revealing minimal Gas6 on the surface of resting human platelets (*c*) and increased levels of Gas6 on human platelets stimulated by ADP 5µM (*d*). ***e*** and ***f***, Flow cytometry of P-selectin on resting platelets (black line) and on ADP-activated platelets (green line), revealing that surface expression of P-selectin increased in stimulated wild-type (*e*) but not in *Gas6*$^{-/-}$ (*f*) platelets. ***g***, Rescue by rGas6 (200 ng/ml) of the defective *Gas6*$^{-/-}$ platelet ATP secretion in response to ADP 20 µM. A representative tracing of ATP secretion by *Gas6*$^{-/-}$ platelets with and without rGas6, and by wild-type platelets is displayed. The average ATP levels (µM) after rGas6 rescue are indicated in Table 1. The arrow indicates the time of application of ADP.

embolism after a collagen/epinephrine challenge *in vivo*. We used antibodies directed against the C-terminal part of Gas6—responsible for binding of Gas6 to its receptors[27]. In contrast to isotype-matched control antibodies, Gas6-neutralizing antibodies dose-dependently blocked aggregation of washed human platelets in response to ADP (5 μM; Fig. 3*l* and *m*), but had no effect in the absence of ADP. Since no rGas6 was exogeneously added, the antibodies blocked Gas6 released from platelet stores and acting extracellularly to stimulate platelet aggregation.

Importantly, Gas6-neutralizing antibodies protected wild-type mice against the fatal collagen/epinephrine-induced thrombo embolism to the same degree (75% survival, $n = 12$; Fig. 2*d*) as genetic loss of Gas6 (80% survival in *Gas6*$^{-/-}$ mice; Fig. 2*c*). Control antibodies were ineffective in preventing fatal thrombo embolism in wild-type mice (25% survival, $n = 16$; Fig. 2*d*). Gas6 antibody-treated mice did not show any signs of bleeding. These results indicate that inhibition of Gas6 effectively blocks thrombosis.

Discussion

Here we provide genetic evidence for a novel role of Gas6 in thrombosis. *Gas6*$^{-/-}$ mice are protected against arterial and venous thrombosis, but do not suffer spontaneous or trauma-induced bleeding. The antithrombotic mechanism of Gas6 deficiency is at least partly due to defective platelet aggregation and secretion. Gas6, though ineffective itself, amplifies the response to known platelet agonists. Neutralizing Gas6 antibodies protect wild-type mice against fatal thrombo embolism without causing spontaneous bleeding.

Gas6$^{-/-}$ mice were resistant to thrombosis as assessed using models known to depend on coagulation and platelets[19,20,28]. Their resistance to thrombosis was not due to differences in coagulation, fibrinolysis or megakaryopoiesis, but to platelet dysfunction. Though ineffective by itself, Gas6 significantly enhanced the formation of stable platelet macro-aggregates in response to several platelet agonists. In the absence of Gas6, low concentrations of these agonists could only induce reorganization of actin filaments, responsible for the shape change preceding initial platelet micro-aggregation. Signaling by the ADP, collagen, TXA_2 or thrombin receptors was not completely blocked in *Gas6*$^{-/-}$ platelets, as a shape change did occur in response to low concentrations and irreversible platelet aggregation proceeded in response to high concentrations. Only thrombin induced aggregation of *Gas6*$^{-/-}$ platelets at low concentrations, but these aggregates were smaller, loosely packed and incompletely degranulated. Thus, secretion and aggregation of platelets could occur, but both were less efficient in *Gas6*$^{-/-}$ platelets. As secretion of ADP is essential to secure formation of stable platelet macro-aggregates, only unstable *Gas6*$^{-/-}$ platelet microaggregates formed at low agonist concentration. Higher concentrations of platelet agonists or a potent agonist like thrombin were required to induce formation of stable *Gas6*$^{-/-}$ platelet macro-aggregates. An additional mechanism that could contribute to the defective platelet aggregation in *Gas6*$^{-/-}$ mice may relate to the reduced formation of fibrinogen bridges linking adjacent activated platelets.

An autocrine role for Gas6 in platelets is indicated by the finding that Gas6 is present in α-granules and, following platelet activation, becomes secreted and bound to Gas6 receptors. As *Gas6*$^{-/-}$ platelets have normal expression of the Gas6 receptors Axl or Sky, the platelet defects were not related to downregulation of these receptors. Collectively, our data are consistent with a model where Gas6 is released from the α-granules upon initial stimulation of platelets by several agonists. Subsequently, Gas6 amplifies—by signaling through one or more of its receptors—the intracellular signals generated from the ADP, collagen, TXA_2 and thrombin receptors. Gas6 might exert this amplification signal at the level or downstream of the platelet agonist receptors, but most likely upstream of protein kinase C activation or Ca^{++} mobilization. Indeed, the downstream pathways mediating granule secretion and platelet aggregation[18] were functional in *Gas6*$^{-/-}$ platelets, since PMA or the Ca^{++} ionophore A23186 induced normal secretion and aggregation.

Consistent with Evenas *et al.*[21], Gas6 does not seem to proteolytically activate existing coagulation factors, but we cannot exclude the possibility that Gas6 triggers expression of some essential coagulation factors. Indeed, Gas6 could also activate leukocytes and endothelial cells, as these cells express Gas6 receptors[29,30]. Gas6 might also assist platelet aggregation by physically linking platelets together, for example its C-terminal G-domain could bind a Gas6 receptor on one platelet and form a bridge to another platelet via binding of its N-terminal Gla-domain to phospholipids on an adjacent platelet. This cell-adhesion activity of Gas6 may assist, but does not appear to mediate, platelet aggregation, as Gas6 by itself was unable to induce platelet aggregation. In addition, *Gas6*$^{-/-}$ platelets formed aggregates when stimulated by high concentrations of agonists.

Congenital abnormalities in platelet aggregation and secretion have been identified in a number of patients suffering from mild bleeding syndromes[18]. Some of these patients may have a 'signal transduction defect'[18]. Like *Gas6*$^{-/-}$ mice, patients with primary signaling transduction defects have impaired secretion of dense granules in response to weak agonists or to low concentrations of potent agonists. Fibrinogen levels on platelets are also reduced in these patients, but their number of platelet granules, TXA_2 production and initial aggregation are normal[31]. The present investigation indicates that Gas6 defects might constitute a possible mechanism of some of these primary signal transduction defects.

A function for Gas6 or its receptors in thrombosis has not been demonstrated previously. Deficiency of the purinergic P2Y1 receptor[32,33] or of GTP-binding Gαq (ref. 22) displays a comparable protection against fatal collagen-induced thrombo embolism. However, in contrast to these other mouse models, Gas6 deficiency did not increase bleeding time after tail clipping. Thus, Gas6 appears to be redundant for baseline hemostasis, but constitutes an important 'amplification' system in pathological conditions. Precisely because Gas6 only amplifies the response of other platelet agonists—while not evoking a response itself—inhibition of Gas6 might constitute an attractive treatment to prevent thrombosis without causing bleeding side effects.

Methods

Generation of *Gas6*$^{-/-}$ mice. We screened a SVJ mouse genomic library in lambda FIX II (Stratagene) with cDNA probes of mouse *Gas6* (from C. Schneider). A total homology of 6.2 kb was used to construct the targeting vector p*PNT.gas6*. R1 embryonic stem cells were electroporated with NotI-linearized p*PNT.gas6*. The correctly targeted ES cell clones were used for aggregation with Swiss morula embryos to generate chimeric animals, which were test bred for germline transmission, and the resulting heterozygous mice intercrossed to obtain homozygous offspring. Genotyping was performed by Southern-blot analysis or PCR amplification of mouse tail DNA using allele-specific probes. Housing and procedures involving experimental animals were approved by the Institutional Animal Care and Research Advisory Committee of the University in Leuven[34].